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Inhibitors of dihydro-orotase, amidophosphoribosyltransferase and IMP cyclohydrolase as potential drugs

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The *de novo* pathways for the biosynthesis of pyrimidine and purine nucleotides provide the deoxynucleoside triphosphates (dNTPs) required for DNA synthesis. A specific inhibitor of an enzyme of the pyrimidine or purine pathways may induce in growing cells a deficiency of a particular dNTP required for DNA synthesis, or an imbalance in the cellular pools of dNTPs (dCTP, dTTP, dATP and dGTP) leading to genetic miscoding [1]. Such inhibitors may have anti-cancer activity [2], and inhibitors of *de novo* pyrimidine biosynthesis may be effective anti-malarial drugs [3]. Inhibitors of *de novo* nucleotide biosynthesis such as the glutamine antagonist acivicin [4] and the anti-folate methotrexate [5] lack specificity and may inhibit several reactions, including those catalysed by enzymes not involved in DNA synthesis. Acivicin and methotrexate produce some side-effects in patients, although the latter is a very useful anti-cancer drug. The rational design and synthesis of metabolite analogues which are potent and specific inhibitors of a particular enzyme-catalysed reaction should lead to the development of anti-cancer (and anti-malarial) drugs with fewer side-effects and the potential for greater control of cytotoxity. We have studied the enzymes dihydro-orotase (EC 3.5.2.3), amidophosphoribosyltransferase (EC 2.4.2.14) and IMP cyclohydrolase (EC 3.5.4.10) with these objectives.

Abbreviations used: AICAR, 5-aminoimidazole-4-carboxamide ribotide; CA-asp, *N*-carbamyl-L-aspartate; DHO, L-dihydro-orotate; FAICAR, 5-formamidoimidazole-4-carboxamide ribotide; GAR, glycinamide ribotide; HDDP, 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylate; IMP synthase, the bifunctional enzyme which contains AICAR transformylase and IMP cyclohydrolase activities; pe, parasitized erythrocyte; PRA, phosphoribosylamine; P-Rib-PP, 5-phosphoribosyl-1-pyrophosphate; PTX, piritrexim; TDHO, thiodihydro-orotate.

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The catalytic mechanism of dihydro-orotase

The *de novo* pathway for the biosynthesis of pyrimidine nucleotides consists of nine reactions leading to CTP (Scheme 1). The first three reactions are catalysed by a trifunctional enzyme called dihydro-orotate (DHO) synthetase or CAD. Dihydro-orotase catalyses the third reaction [*N*-carbamyl-L-aspartate (CA-asp)→DHO] and is the central domain of trifunctional CAD [6]. Christopherson and Jones [7] proposed that dihydro-orotase has a zinc atom at the active site with a catalytic role similar to that seen in the zinc proteases; the presence of zinc in the dihydro-orotase domain was subsequently confirmed [8]. The enzyme is inhibited by the zinc chelator L-cysteine [7] and the pH–activity profiles for the biosynthetic and degradative reactions are 'mirror images', consistent with ionization of a catalytic residue of the enzyme–substrate complex with a pK_a of 7.1 (Zn^{II}-OH_2 [9]). Chemical modification experiments with diethyl pyrocarbonate [9] and citraconic anhydride [10] provided evidence for the presence of histidine and lysine residues(s) respectively at the active site of dihydro-orotase.

We have subcloned the cDNA encoding the hamster dihydro-orotase domain using selection by complementation of an *Escherichia coli pyr C*

Scheme 1

Pathway for the biosynthesis of pyrimidine nucleotides

CAP, carbamyl phosphate; Oro, orotate; OMP, orotidine monophosphate. See the text for further details.

$HCO_3^- \rightarrow$CAP→CA-asp→DHO→Oro→OMP→UMP→UDP→UTP→CTP

mutant lacking dihydro-orotase activity [6]. The active recombinant dihydro-orotase domain was expressed as an enzyme of 39 kDa [11]. The recombinant dihydro-orotase was purified and shown to have catalytic properties similar to those of the native domain. The amino acid sequence of the hamster dihydro-orotase domain, numbered from the N-terminal Thr-1 of the domain liberated by proteolysis of trifunctional CAD with elastase, contains five His residues which are totally conserved in the eight known dihydro-orotase sequences (His-15, -17, -158, -186 and -234 [12]); several other His residues are partially conserved (e.g. His-134 and -185). To determine which His residues co-ordinate the zinc atom at the active site of dihydro-orotase, several mutant enzymes were prepared (His15→Gly, His17→Gly, His134→Gly, His158→Ala, His186→Ala and His234→Ala) and transformed strains of *E. coli* were grown in the presence of $^{65}ZnCl_2$. The recombinant dihydro-orotases mutated at His-15, -17 and -158 did not bind ^{65}Zn, the mutant at His-186 showed a reduced affinity for ^{65}Zn while mutants at His-134 and His-234 bound ^{65}Zn like the wild-type enzyme [12,13]. These data indicate that the three zinc ligands are His-15, -17 and -158. Incubation of dihydro-orotase with diethyl pyrocarbonate would probably result in the formation of covalent adducts with several of these His residues.

Alignment of the eight known dihydro-orotase sequences has revealed four conserved regions [6] containing some conserved amino acid residues that are important for enzyme function. The most highly conserved region is near the N-terminal Thr-1 of the domain and shows identity with nonapeptides from the active sites of carbonic anhydrases (Figure 1), where the two His residues are known to co-ordinate a zinc atom which participates in catalysis [14]. The ^{65}Zn binding experiments described above implicated His-15, His-17 and His-158 as zinc ligands, and the sequence identity with carbonic anhydrase supports this conclusion. His-94 of carbonic anhydrase is hydrogen-bonded to the amide carbonyl of Gln-92, forming a carbonyl–His–zinc triad [15]. This triad has been identified in all zinc enzymes of known three-dimensional structure, including carbonic anhydrase, and is likely to occur in dihydro-orotase. The carbonyl group of Asp-13 could hydrogen-bond to His-15, which in turn co-ordinates the zinc ($C{=}O...His{\rightarrow}Zn^{II}$). Site-directed mutagenesis of Asp-13→Asn or Arg-19→Lys leads to completely inactive mutant enzymes [10,12,13]. The conserved Arg-19 of this nonapeptide is likely to form an electrostatic bond with the 4-carboxylate of DHO bound at the active site. Proposed roles for the four conserved amino acid residues of the nonapeptide, i.e. Asp-13, His-15, His-17 and Arg-19, are shown in Figure 2. Among the 22 amino acid residues which are totally conserved between the eight dihydro-orotase sequences there is one lysine, Lys-239, which may have reacted with citraconic anhydride in the modification experiment described above. Mutant recombinant dihydro-orotases with the substitutions Lys-239→Arg and Lys-239→Gly were prepared, purified and the catalytic properties compared with those of the wild-type enzyme ($K_m = 4.02\ \mu M$; $V_{max} = 1.15\ \mu mol/min$ per μg [12,13]). The conservative substitution Lys-239→Arg increased the K_m for DHO 3.9-fold without a significant effect upon the $V_{max.}$, while Lys-239→Gly increased the K_m value 110-fold and the $V_{max.}$ 2.1-fold. The 110-fold increase in K_m observed on loss of the basic Lys residue is consistent with the loss of a hydrogen bond with DHO.

A proposed catalytic mechanism for the reverse reaction catalysed by dihydro-orotase (DHO→CA-asp) is shown in Figure 2, and parallels the mechanism originally proposed by Christopherson and Jones [7]. DHO binds at the active site (step I) with the C-6 carbonyl close to the zinc but interacting with a His, perhaps His-234, and the C-4 carboxylate interacting with Arg-19. The C-2 carbonyl forms a hydrogen bond with Lys-239. Catalysis is initiated by nucleophilic attack by the zinc-bound hydroxide at the polarized carbonyl group of C-6 (step II). The tetrahedral intermediate formed (step III) is stabilized by co-ordination of the two oxygens to the zinc and by the positively charged His. An amino acid residue, perhaps Asp-230, donates a

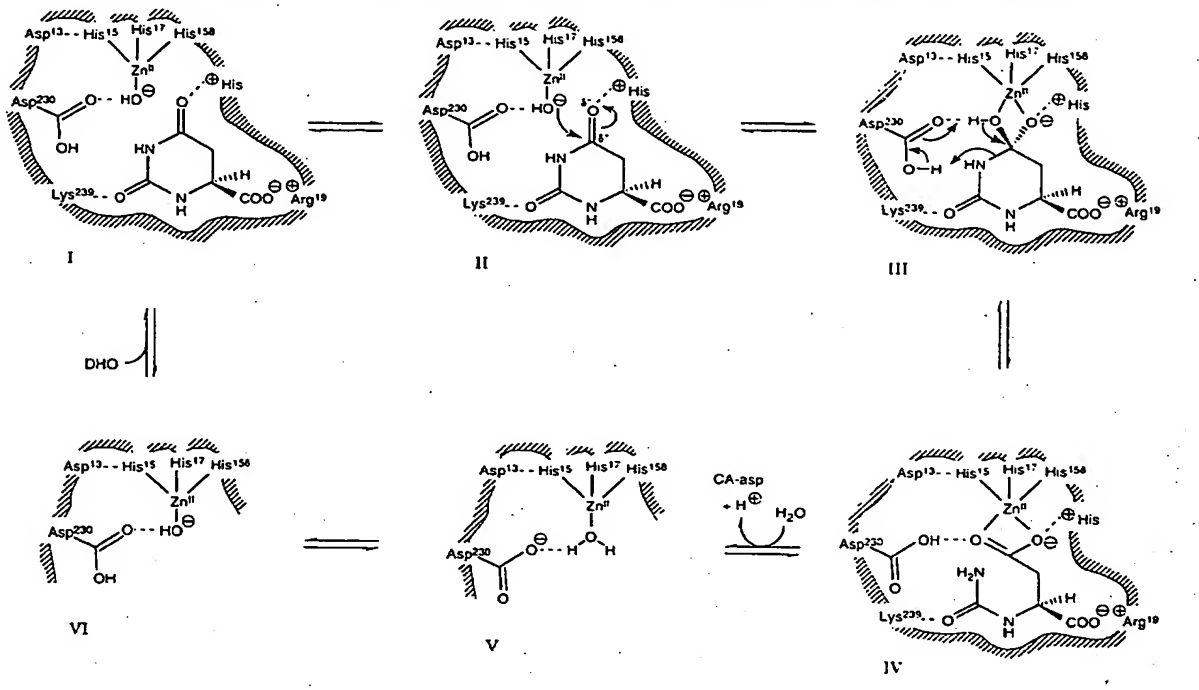
Figure 1

Identity between dihydro-orotase and carbonic anhydrase

Hamster dihydro-orotase	12	Ile-Asp-Val-His-Val-His-Leu-Arg-Glu
Human carbonic anhydrase II	91	Ile-Gln-Phe-His-Phe-His-Trp-Gly-Ser

Figure 2

Proposed mechanism for the hydrolysis of DHO to CA-asp catalysed by hamster dihydro-orotase



proton to the ureido N-1 atom, and abstracts a proton from the new hydroxy group at C-6, to form CA-asp (step IV). The product, in this case CA-asp, dissociates, and the active site reverts to its Zn^{II}-OH^- form (step VI), ready for another catalytic cycle.

With a knowledge of the catalytic mechanism of dihydro-orotase (Figure 2), we have designed and synthesized a series of inhibitors of dihydro-orotase. 6-L-Thiodihydro-orotate (TDHO; $K_i = 0.85\ \mu M$) and 2-oxo-1,2,3,6-tetrahydropyrimidine-4,6-dicarboxylate (HDDP; $K_i = 0.74\ \mu M$) are potent inhibitors of hamster dihydro-orotase [16]. The methyl esters of TDHO and HDDP more readily enter human CCRF-CEM leukaemia cells, and their IC_{50} values are 25 μM and 21 μM respectively [17]. However, inhibitors of *de novo* pyrimidine biosynthesis have not been useful as anti-cancer drugs, due perhaps to the roles of pyrimidine nucleotide precursors in RNA, polysaccharide and lipid biosyntheses.

The malarial parasite, *Plasmodium falciparum*, can only synthesize pyrimidine nucleotides via the *de novo* pathway [18] (Scheme 1), while humans have the *de novo* pathway and an alternative salvage pathway from preformed precursors such as uridine and cytidine. It therefore follows that specific inhibitors of the *de novo* pathway will have a selective toxicity against malaria. We have found that TDHO methyl ester has an IC_{50} value of approx. 35 μM against *P. falciparum* growing in erythrocytic culture. We also found that TDHO methyl ester (250 μM; 6 h) induces accumulation of CA-asp from 1.9 to 120 amol/parasitized erythrocyte (pe), and of DHO from undetectable levels to 12.5 amol/pe, with a decrease in total pyrimidine nucleotides (UMP + UDP-sugars + CDP + UDP + CTP + UTP) to 57% [3]. TDHO does inhibit dihydro-orotase (CA-asp→DHO) in growing malaria but there is also some inhibition of DHO dehydrogenase (DHO→orotate), indicated by the accumulation of some DHO in treated parasites. Addition of orotate with TDHO partially relieved the toxicity of TDHO; some of the toxicity may be due to the marked accumulation of CA-asp observed and to several possible metabolites of CA-asp which also accumulated. We have found that, in parasites treated with TDHO methyl ester (250 μM; 6 h), dTTP (0.38 amol/pe) decreased to 18% of control, dCTP (0.24 amol/

pe) and dATP (0.56 amol/pe) showed only minor changes while dGTP was not detectable (K. K. Seymour and R. I. Christopherson, unpublished work). TDHO induces a major deficiency in dTTP in malaria, and this imbalance in dNTPs could result in genetic miscoding and consequent cytotoxicity.

An inhibitory allosteric site for dihydrofolate and anti-folates on amidophosphoribosyltransferase

The *de novo* pathway for the biosynthesis of purine nucleotides consists of 10 reactions leading to IMP (Scheme 2). Amidophosphoribosyltransferase catalyses the first reaction of the pathway [5-phosphoribosyl-1-pyrophosphate (P-Rib-PP)→phosphoribosylamine (PRA)] and is subject to feedback inhibition by the purine nucleoside 5′-monophosphates IMP, AMP and GMP [19], which were thought to bind at an allosteric site(s). The enzyme exhibits positive co-operativity with respect to the substrate, P-Rib-PP [20] and the purine nucleotides promote this co-operativity [21]. Amidophosphoribosyltransferase conforms to an ordered sequential mechanism, with P-Rib-PP binding first followed by L-glutamine [22]. An additional mechanism of regulation of amidophosphoribosyltransferase has been discovered in our laboratory [5]. Mouse L1210 leukaemia cells were grown in the presence of [^{14}C]glycine which was incorporated into purine intermediates at reaction 2, catalysed by glycinamide ribotide (GAR) synthetase (PRA→GAR; Scheme 2), with prominent ^{14}C-labelling of the abundant purine nucleotides (ADP, GDP, ATP and GTP). Methotrexate was thought to induce inhibition of the purine pathway at the reaction catalysed by 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase [AICAR→5-formamidoimidazole-4-carboxamide ribotide (FAICAR); Scheme 2] [23]. However, when L1210 cells were pulse-labelled with [^{14}C]glycine for 2 h at various times relative to the addition of 0.1 μM methotrexate, there was only minor accumulation of GAR and AICAR 2 h after addition of the anti-folate, but no ^{14}C-labelled purine intermediates accumulated after 8 h [5]. Methotrexate is a potent inhibitor of dihydrofolate reductase, resulting in accumulation of dihydrofolate polyglutamates [24] which inhibit GAR and AICAR transformylases (GAR→FGAR [25]; AICAR→FAICAR [26]; Scheme 2). These observations strongly suggested that the primary site of inhibition was upstream in the purine pathway, perhaps at reaction 1 catalysed by amidophosphoribosyltransferase (P-Rib-PP→PRA; Scheme 2). In support of this hypothesis, 0.1 μM methotrexate induced a 3.4-fold accumulation of P-Rib-PP in L1210 cells after 3 h. It therefore seemed likely that dihydrofolate polyglutamates, which accumulated in response to methotrexate, were inhibiting amidophosphoribosyltransferase via an unreported mechanism. Experiments *in vitro* with partially purified amidophosphoribosyltransferase showed that dihydrofolate-Glu_5 is a non-competitive (allosteric) inhibitor of the enzyme with a dissociation constant of 3.4 μM for interaction with the enzyme–glutamine complex [5]. Dihydrofolate polyglutamates accumulate to 20% of the total folate pool in methotrexate-treated cells [24]. The total cellular concentration of reduced folates in L1210 cells is about 10 μM [27], suggesting that dihydrofolate polyglutamates would accumulate to a concentration of about 2 μM after methotrexate addition, sufficient to inhibit amidophosphoribosyltransferase [5]. The non-classical anti-folate, piritrexim (PTX), is also a potent inhibitor of *de novo* purine biosynthesis and amidophosphoribosyltransferase with a dissociation constant of 6.0 μM [5]. In normal cells, inhibition of amidophosphoribosyltransferase by dihydrofolate polyglutamates would signal the unavailability of C_1 derivatives of tetrahydrofolate for reactions 3 and 9 of purine biosynthesis (Scheme 2).

The purine nucleotides IMP, AMP and GMP induce formation of an inactive tetramer (224 kDa) of amidophosphoribosyltransferase from an active dimer (112 kDa) found in the presence of P-Rib-PP [22,28]. Using sucrose density centrifugation, we showed that piritrexim induces formation of an inactive dimer (112 kDa; 7.2 S) from the active dimer (6.7 S) as shown in Scheme 3. Inhibition patterns for PTX and AMP have been obtained as a function of P-Rib-PP and L-glutamine concentrations and have been fitted to appropriate velocity equations. We have

Scheme 2

Pathway for the biosynthesis of purine nucleotides

FGAR, N-formylglycineamide ribotide; FGAM, N-formylglycineamidine ribotide; AIR, 5-aminoimidazole ribotide; CAIR, 4-carboxy-5-aminoimidazole ribotide; SAICAR, N-succino-5-aminoimidazole-4-carboxamide ribotide. See the text for further details.

P-RiB-PP→PRA→GAR→FGAR→FGAM→AIR→CAIR→SAICAR→AICAR→FAICAR→IMP

found that PTX, like pentaglutamyl dihydrofolate, is a potent non-competitive (or allosteric) inhibitor of amidophosphoribosyltransferase which is bound with positive co-operativity. The dissociation constant for binding the first molecule of PTX is 66 μM, while that for the second is only 12 μM, indicating a much tighter second interaction. Inhibition patterns for AMP consistently intersected on the $1/v$ ordinate, indicating that AMP is a competitive inhibitor with respect to P-Rib-PP of amidophosphoribosyltransferase, which binds without co-operativity with a dissociation constant of 44 μM. AMP was thought to be an allosteric inhibitor of the enzyme, and our conclusions certainly supersede this concept.

Potent inhibitors of IMP cyclohydrolase

Reactions 9 and 10 of the *de novo* purine pathway (AICAR→FAICAR→IMP; Scheme 2) are catalysed by a bifunctional enzyme called IMP synthase. We have purified this enzyme, with a subunit molecular mass of 62.1 kDa, 780-fold from human CCRF-CEM leukaemia cells [29]. Using a sensitive radioassay [30], IMP cyclohydrolase has a K_s value for FAICAR at pH 7.4 of 0.87 μM. The pH-dependencies of the $V_{max.}$ and $V_{max.}/K_s$ values for IMP cyclohydrolase are consistent with a single ionizable amino acid residue with a pK_a of 7.6 which must be in the basic form for binding of FAICAR and subsequent catalysis. Chemical modification of IMP cyclohydrolase with *N*-ethylmaleimide and phenylglyoxal provided evidence for the presence of cysteine and arginine residues at the active site [29]. The arginine may form an electrostatic bond with the negatively charged phosphate group of FAICAR. The thiolate form of a cysteine residue at the active site with a pK_a of 7.6 may attack the 5-formamido carbon of FAICAR with formation of a tetrahedral oxyanion transition state (Figure 3). This mechanism bears some resemblance to a reversal of the mechanism for a thiol protease [31].

We tested 19 purine nucleoside 5′-monophosphate derivatives as inhibitors of IMP cyclohydrolase and found six which acted as competitive inhibitors, with K_i values of less than 2 μM (Figure 3). The chemical structures of these inhibitors provide valuable information

Scheme 3

Conformations of amidophosphoribosyltransferase (amido-PRTase)

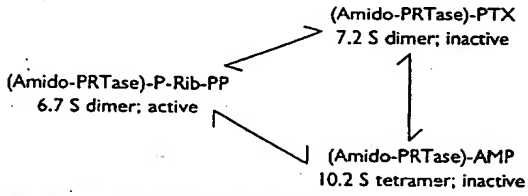
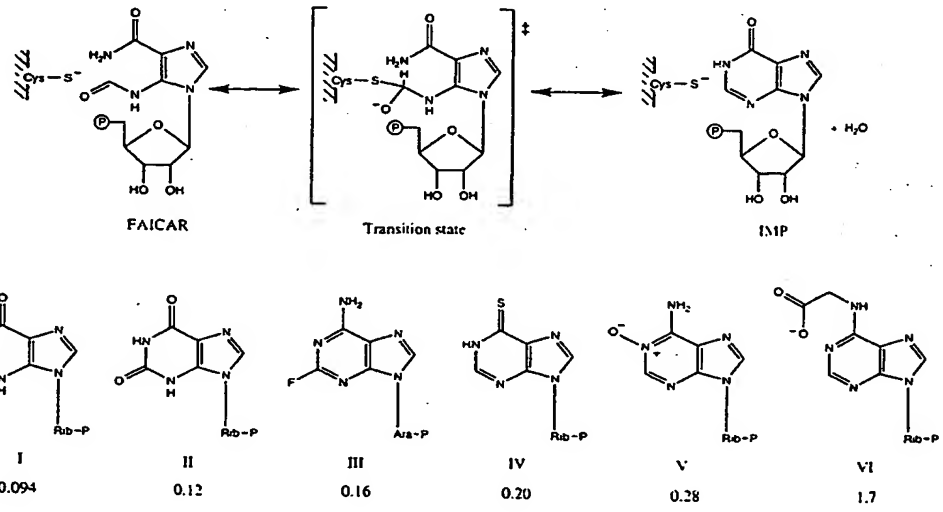


Figure 3

Reaction catalysed by IMP cyclohydrolase and potent competitive inhibitors

I, 2-mercaptoinosine 5′-monophosphate; II, xanthosine 5′-monophosphate (XMP); III, 2-fluoroadenine arabinoside 5′-monophosphate; IV, 6-mercaptopurine riboside 5′-monophosphate; V, adenosine N^1-oxide 5′-monophosphate; VI, N^6-(carboxymethyl)adenosine 5′-monophosphate.



about the structural requirements for binding at this active site. The purine heterocyclic structure is required with a ribose or arabinose 5′-monophosphate group. An electronegative substituent such as sulphur, oxygen or fluorine in the 2-position on the purine ring with an oxygen or amino group in the 6-position gave the most potent inhibition (Figure 3). All of the potent inhibitors have electronegative substituents in the 6-position.

The most potent inhibitors were 2-mercaptoinosine 5′-monophosphate ($K_i = 0.094\ \mu M$) and XMP ($K_i = 0.12\ \mu M$), which is a precursor of GMP (IMP→XMP→GMP). Inhibition of IMP cyclohydrolase by accumulated XMP in growing cells deficient in L-glutamine required for the XMP→GMP transition could limit the further synthesis of XMP. Two of the inhibitors, 2-fluoroadenine arabinoside 5′-monophosphate (Fludarabine; $K_i = 0.16\ \mu M$) and 6-mercaptopurine riboside 5′-monophosphate ($K_i = 0.20\ \mu M$; Figure 3) are derivatives of anti-cancer drugs, but none of the six purine derivatives as the equivalent nucleosides induced inhibition of IMP cyclohydrolase in growing leukaemia cells [29].

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